Supporting Information

Supporting Information 1: *foxA cis*-regulatory modules and the binding sites within them

(A) Module *F* is located -10780 to -9489 upstream of *foxA* translation start and contains one Tcf functional binding site highlighted in green and one Su(H) site highlighted in cyan. (B) Module *I* is located -1835 to -1363 upstream of *foxA* translation start and contains one Foxa functional binding site highlighted in magenta and one Brachyury functional binding site highlighted in purple. (C) Module *J* is located immediately upstream of the translation start site and contains two Su(H) functional sites highlighted in cyan and two functional Otx sites highlighted in blue. (D) Region *K* is located +3127 to +6820 downstream the start of translation and contains three functional Hox11/13b site highlighted in brown and ten putative Otx sites highlighted in blue.

A. Module F

ACTTGACGCTTGGACGAAAAAAGAACACAAGTCATAAATGAGATATAGGTCAGTCATAA
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B. Module I

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C. Module J

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D. K Region

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Supporting Information 2: Modeling the effect of a binding site mutation on its target's expression level

Here we simulate the effect of a binding site mutation of a transient input on the target expression level. For simplicity we assume that gene *Z* is activated by an activator, X, and a transient input Y, Fig. 4A. The expression of gene *X* starts at *t=0.* Gene *Y* turns on about 3 hours after *X* is turned on, and its transcription is shut off 6 hours later. We assume that the *cis*-regulatory module of gene *Z* applies additive OR logic on its two inputs. We used the following set of differential equations to describe this circuit (1-3):

$$(1)\ \frac{dmX(t)}{dt} = I_0 - k_{dm} mX(t),$$

$$(2)\ \frac{dX(t)}{dt} = k_t mX(t) - k_{dP} X(t),$$

$$(3) Trans(t) = \begin{cases} 0, t < 180, t > 540, \\ Y_0, 180 < t < 540. \end{cases}$$

$$(4)\ \frac{dmY(t)}{dt} = I_{\max}\left(1 - \exp\left(-\frac{k_{bTrans} Ocp_{Trans}(t - T_m)}{I_{\max}}\right)\right) - k_{dm} mY(t),$$

$$(5)\ \frac{dY(t)}{dt} = k_t mY(t) - k_{dP} Y(t),$$

$$(6)\ \frac{dmZ(t)}{dt} = \frac{I_{\max}}{2}\left\{\left(1 - \exp\left(-\frac{k_{bX} Ocp_X (t - T_m)}{I_{\max}}\right)\right) + \left(1 - \exp\left(-\frac{k_{bY} Ocp_Y (t - T_m)}{I_{\max}}\right)\right)\right\} - k_{dm} mZ(t),$$

$$(7)\frac{dZ(t)}{dt} = k_t mZ(t) - k_{dP}Z(t).$$

$$(8)Ocp_P = \frac{K_r P(t)}{D_n + K_r P(t)}.$$

Here *mX(t)* , *mY(t)* and *mZ(t)* are the number of mRNA molecules per cell of genes *X*, *Y* and *Z* respectively. *X(t)*, *Y(t)* and *Z(t)* are the number of protein molecules per cell of genes *X*, *Y* and *Z* respectively. *Trans(t)* is a mathematical function that introduce the time window in which gene *Y* is active in the cell. $Ocp_p(t)$ is the occupancy of the binding site of the transcription factor P, where P can be either X, Y or Trans. The transcription initiation rates are the following: $I_0=1$, $I_{max}=11$ *mRNA/min.* The level of Trans when it is active is $Y_0= 25000$. The kinetic parameters are the following: mRNA and protein turnover rates for all genes: $K_{dm} = K_{dP} = 0.005\ min^{-1}$, which means a half life of about 2 hours. The transcriptional delay is $T_m=20$ *min*. The translation rate, K_t = 2 protein/(min × mRNA). The activation strength for all transcription factors is, $K_b=5$. The equilibrium constant $K_r=10^5$ and the available genome $D_n= 7.2\times10^8$, assuming that the open chromatin is about a 90% of the total sea urchin genome, which is 8×10^8. The results of this simulation are presented in Fig. 4.

1. Ben-Tabou de-Leon S & Davidson EH (2009) Modeling the dynamics of transcriptional gene regulatory networks for animal development. *Dev Biol* 325(2):317-328.
2. Bolouri H & Davidson EH (2003) Transcriptional regulatory cascades in development: initial rates, not steady state, determine network kinetics. *Proc Natl Acad Sci U S A* 100(16):9371-9376.
3. Davidson EH (1986) *Gene Activity in Eraly Development.* (Academic Press. Inc., Orlando, Florida); Third Ed.

Table S1: Binding site mutations

Transcription factor	**Intact site**	**Mutated site**
Tcf site in *F*	TCAAAG	GTCCCA
Otx site in *F*	TAATCT	CCCGAG
1st Otx site in *J*	TAATCT	CCCGAG
2nd Otx site in *J*	GGATTA	CCCGAG
Su(H) site in *F*	ATCTCATA	ACCTTTCG
1st Su(H) site in *J*	TTCTCATA	TAGTAGTA
2nd Su(H) site in *J*	TGTGGGAG	GCCTTTCG
Foxa site in *I*	TTGTTTAT	TTAGGGTT
Brachyury site in *I*	AGGTGCGACAAT	TTAACAGTCCCG
1st Hox11/13b site in K	GTAATAAA	AGCGTCCA
2nd Hox11/13b site in K	TAATAATAAT	TGGTCCTGCT
3rd Hox11/13b site in K	GTAATAAA	AGCAGCAC

Table S1 presents the point mutations that were generated for the different transcription factor binding sites in each *cis*-regulatory module.

Table. S2: Mutation and perturbation analysis results and statistics

Mutation/Perturbation	Time	# of Repeats	Average ratio to WT	Standard Error	p-value in z-test
Δ×3 Su(H) (FIJ)	**15hpf**	**2**	**0.63**	**8%**	**$<10^{-6}$**
Δ×3 Su(H) (FIJ)	**18-19hpf**	**2**	**0.61**	**2%**	**$<10^{-6}$**
Δ×3 Su(H) (FIJ)	24hpf	3	0.7	26%	0.12
Δ×3 Hox11/13 (F to K)	15hpf	2	0.65	24%	0.17
Δ×3 Hox11/13 (F to K)	**18-19hpf**	**4**	**0.55**	**9%**	**$<10^{-6}$**
Δ×3 Hox11/13 (F to K)	**24hpf**	**4**	**0.53**	**7%**	**$<10^{-6}$**
Δ Brachyury (FIJ)	18-19hpf	3	0.86	21%	0.25
Δ Brachyury (FIJ)	**20-21hpf**	**3**	**0.65**	**4%**	**$<10^{-6}$**
Δ Brachyury (FIJ)	**24hpf**	**3**	**0.43**	**1%**	**$<10^{-6}$**
Δ Brachyury (FIJ)	**27hpf**	**3**	**0.63**	**14%**	**0.003**
Δ Foxa (FIJ)	18-19hpf	2	1.27	28%	0.17
Δ Foxa (FIJ)	**20-21hpf**	**2**	**1.34**	**16%**	**0.009**
Δ Foxa (FIJ)	**24hpf**	**2**	**1.99**	**41%**	**0.008**
Δ Foxa (FIJ)	27hpf	2	1.25	50%	0.19
Δ×3 Otx (FIJ)	24hpf	4	1.00	20%	0.5
Δ×3 Otx (FIJ)	**27hpf**	**3**	**0.65**	**9%**	**$<10^{-6}$**
Δ×2 Otx (J)	24hpf	2	1.13	3%	10^{-6}
Δ×2 Otx (J)	**27hpf**	**2**	**0.73**	**8%**	**$<10^{-6}$**
Otx MASO on FIJ	24hpf	2	0.87	26%	0.32
Otx MASO on J	24hpf	1	1.17	NA	NA
Otx MASO on F to K	**24hpf**	**2**	**0.22**	**4%**	**$<10^{-6}$**

Table S2 presents the results and statistics of the mutation and perturbation analysis that were conducted in this work. Values are based on multiple independent batches of 150 injected embryos for a time point. Number of repeats (independent batches) for each experiment is indicated in the table. Marked in bold are the effects we consider to be significantly different than the control ($p<0.01$). In case of mutations the control is the expression level of the wild type construct. In case of perturbation the control is the expression level of the reporter construct coinjected with random MO.

Supporting Information Figure Captions

Fig. S1. Pair wise sequence comparison of two BAC sequences containing the *foxA* gene, using the Family Relations II program (1, 2). The horizontal line at the top represents the sequence of the *Strongylocentrotus purpuratus* BAC clone; the ordinate represents *Lytechinus variegatus* BAC clone. Red lines indicate 20-nucleotide stretches of sequence sharing ≥ 95% identity between the two species. *foxA* is a single exon gene. The exon is indicated as a light blue box. The reporter coding sequence and the SV40 3' UTR were knocked-in the *foxa* BAC replacing the first 94bp of *foxa* coding sequence. The regulatory regions that were identified and analyzed in this work are indicated by light orange boxes. Region *J* has a 226 bp overlap with *foxA* exon. The most 3' boundary of module *J* is the translation start site and therefore *J* includes the basal promoter and the transcription start site.

Fig. S2 Quantification of spatial expression driven by *foxa:RFP BAC* and by the construct *FIJ:GFP* injected alone at 23-26hpf. The construct *FIJ:GFP* is a fusion of modules *F*, *I* and *J* in front of GFP coding sequence and SV40 3' UTR, see Fig. S3. Percentages sum to more than 100% as many embryos express in two or more tissue types. Data are based on three replicate experiments totaling 518 embryos for *foxa:RFP BAC* and 245 for *FIJ:GFP*. The number of expressing embryos in presented in the graph key.

Fig. S3. Reporter construct maps. *Foxa* DNA inserts in the constructs were PCR amplified from the *Spfoxa:RFP* or *Spfoxa:GFP* BACs. The construct *J:GFP* spans the genomic region from 940 bp upstream of the translation start site to the translation start site, followed by GFP coding sequence and SV40 3' UTR. The construct *FIJ:reporter* is a fusion of modules *F, I* and *J* in front of either GFP or RFP coding sequence and SV40 3' UTR. The construct *F to J* spans the entire genomic region from the most 5' end of module *F* through the proximal module *J* to the reporter coding sequence and SV40 3' UTR. The construct *F* to *K* spans the entire genomic region from the most 5' end of module F to the most 3' end of module *K*, including the reporter coding sequence and SV40 3' UTR.

Fig. S4. The NSM clearance of the construct *FIJ:GFP* is affected by Notch MO but not by Su(H) mutations at 24-25 hpf. (A) Quantification of spatial expression of *FIJ:GFP* co-injected with 300 µM Random MO or Notch MO. Percentages sum to more than 100% as many embryos express in two or more tissue types. Data are based on two replicate experiments totaling 67 embryos for Random MO and 112 for Notch MO. Number of expressing embryos for each construct is indicated in the graph legend. (B) Quantification of spatial expression of *FIJ:GFP* and of *FIJ:GFP* with the three Su(H) sites mutated. Data are based on two replicate experiments totaling 86 embryos for intact *FIJ:GFP* and 222 for *FIJ:GFP* with Su(H) sites mutated. Number of expressing embryos for each construct is indicated in the graph legend.

Fig. S5 Otx perturbation and mutation analysis. Throughout the figure error bars show ±1SE. Significance was calculated by the one tailed Z-test. One star indicates $p<0.01$, two stars indicate $p<0.001$. Actual values of means and p-values are presented in Table. S2. (A) The effect of Otx homeodomain MO treatment on the endogenous *foxA*

expression (blue) and on expression of injected reporter constructs (green) at 24 hpf. For each injection we present the reduction in the expression level due to the Otx homeodomain MO treatment (300 µM) compared to random MO treatment (300 µM) for both the endogenous *foxA* and the injected reporter construct. The unity bars represent the random MO values and are presented as a guide to the eye. Maps of the constructs are presented in Fig. S3. The constructs *FIJ:GFP* and *J:GFP* do not respond to the Otx MO while the endogenous *foxA* expression in these injections is reduced by about 65%. The expression of the construct *F to K* is reduced by Otx MO treatment by about 80% while the endogenous *foxA* expression is reduced by about 65%. (B) Mutations of two Otx sites in module *J* and one Otx site in module *F* do not affect the expression of the reporter construct *FIJ:GFP* at 24 hpf, but at 27 hpf the expression is reduced by 35%. Similar behavior is observed for the construct *J:GFP* when the two Otx sites in *J* are mutated. (At least two independent repeats were conducted for all data presented except for the response of *J:GFP* to Otx MO that was measured once. For details see Table. S2).

1. Brown CT, Xie Y, Davidson EH, & Cameron RA (2005) Paircomp, FamilyRelationsII and Cartwheel: tools for interspecific sequence comparison. *BMC Bioinformatics* 6:70.
2. Brown CT, *et al.* (2002) New computational approaches for analysis of cis-regulatory networks. *Dev Biol* 246(1):86-102.

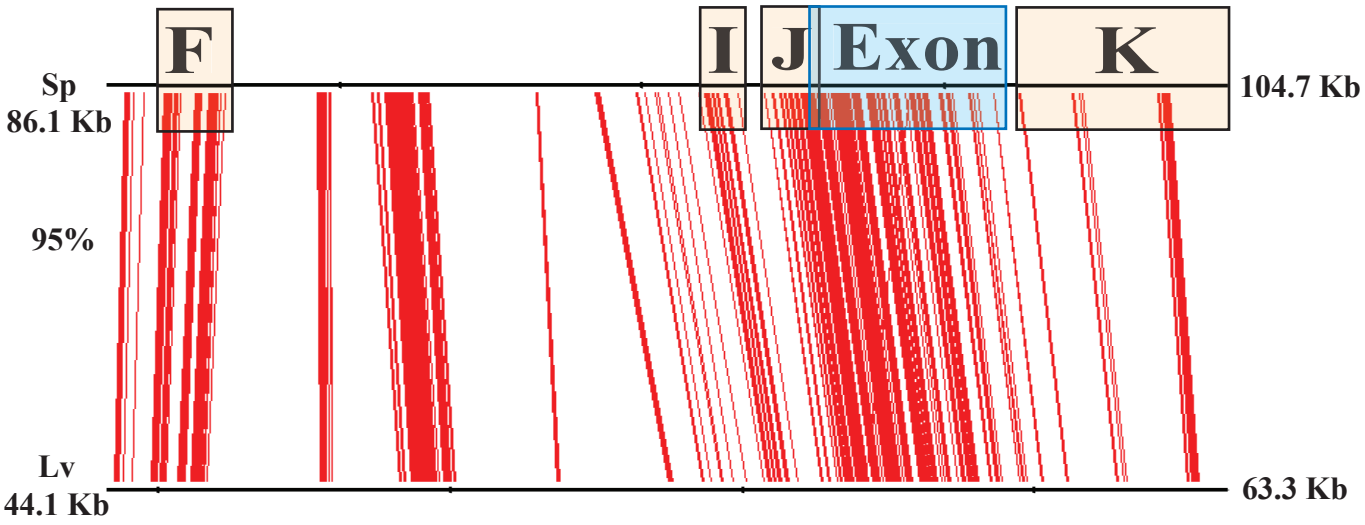
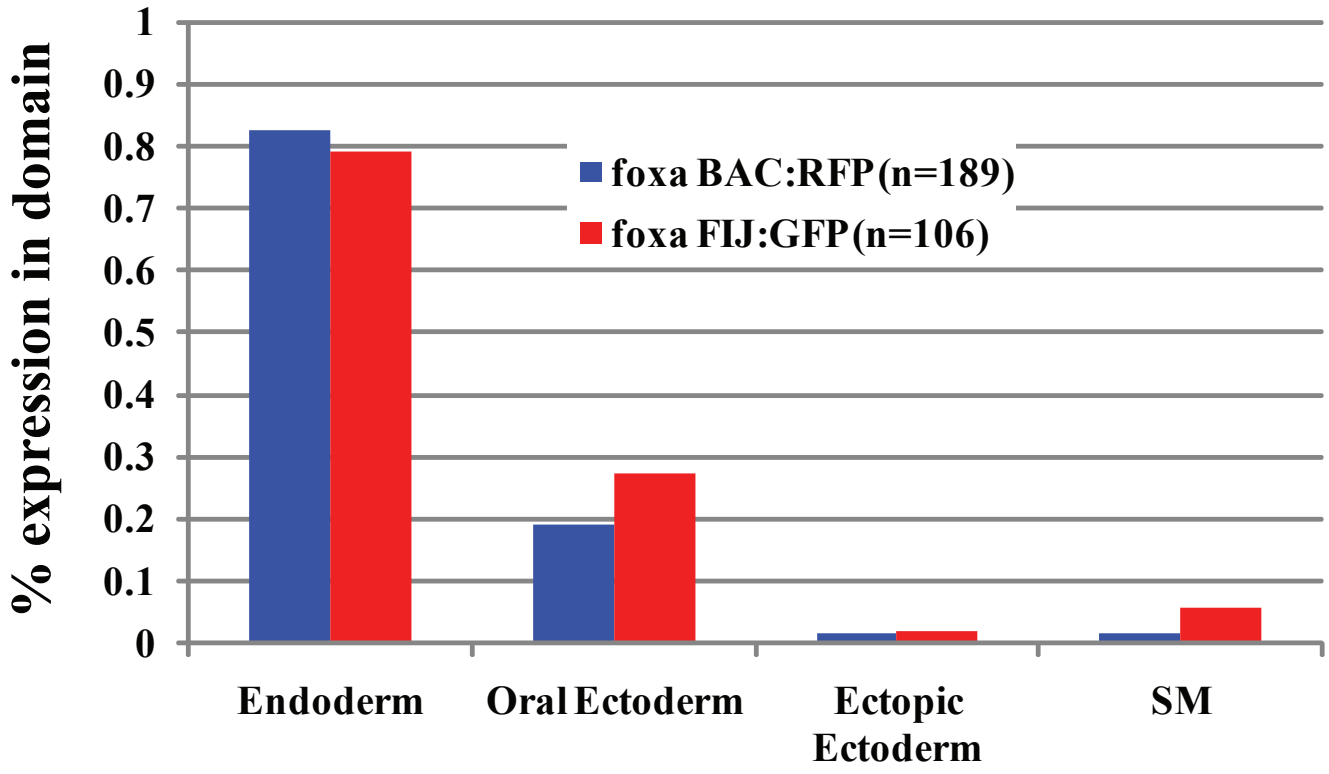


Figure S1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0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
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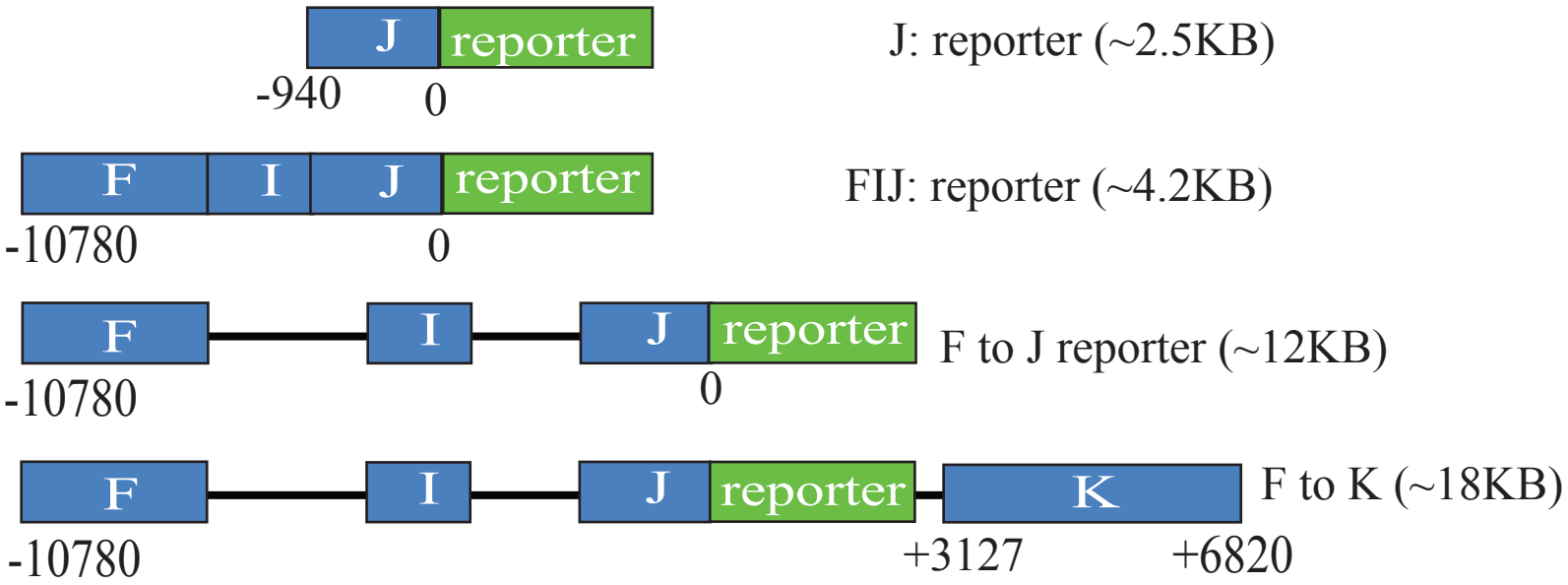
foxa FIJ:GFP (n=106)
Endoderm
Oral Ectoderm
Ectopic Ectoderm
SM

Figure S2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J
reporter
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+3127
+6820
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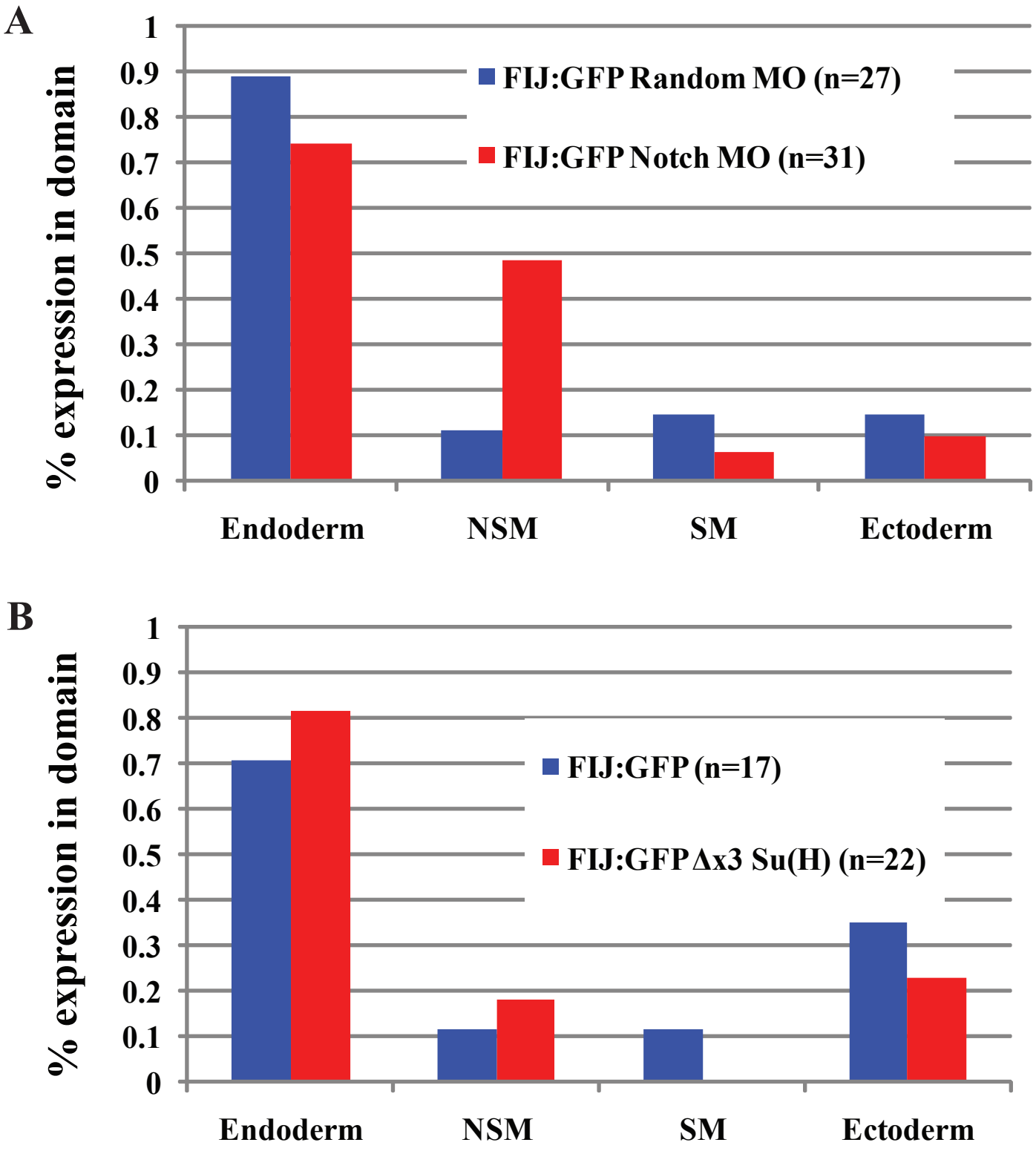
Figure S3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0.8
0.7
0.6
0.5
0.4
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0.2
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FIJ:GFP Notch MO (n=31)
Endoderm
NSM
SM
Ectoderm
B
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0.8
0.7
0.6
0.5
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0.3
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Endoderm
NSM
SM
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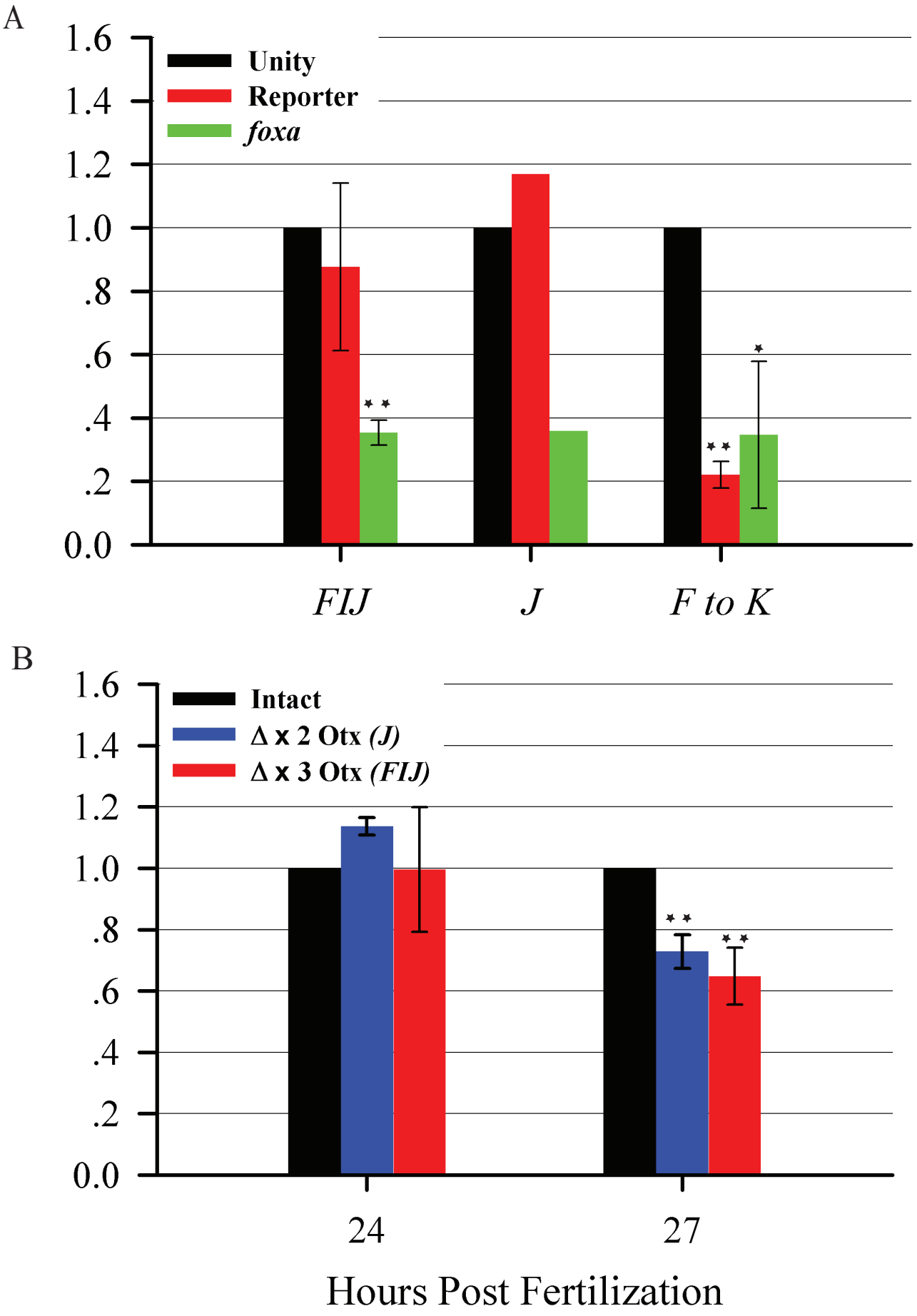
Figure S4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Reporter
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1.0
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.6
.4
.2
0.0
Intact
Δ x 2 Otx (J)
Δ x 3 Otx (FIJ)
24
27
Hours Post Fertilization

Figure S5